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(54) Title: IL-1 INHIBITORS

(57) Abstract

The present invention provides for a novel mechanism of controlling anti-inflammatory activity in a mammal, including humans. The inhibition of P-glycoprotein mediated transport of Interleukin-1 (IL-1) from cells, by an inhibitor of the mdr P-glycoprotein, provides for treatment of indications in which excessive extracellular IL-1 activity is implicated. Such activity has been implicated in such disease as rheumatoid arthritis and osteoarthritis.

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IL-1 INHIBITORS

FIELD OF THE INVENTION

This invention relates to a novel mechanism for inhibition of Interleukin - 1 (hereinafter IL-1) synthesis and hence treatment of disease states in a mammal caused by or exacerbated by IL-1 production.

BACKGROUND OF THE INVENTION

Interleukin-1 is the name given to a family of secreted proteins which have numerous pro-inflammatory properties. These include the stimulation of prostoglandins, endogenous pyrogenic activity, stimulation of immune cell functions, induction of degradative enzymes such as stromelysin, collagenase and various neutral proteases, and induction of other cytokines. These activities are associated with numerous inflammatory conditions, such as rheumatoid arthritis and inflammatory bowel disease, inter alia, thus implicating IL-1 as a major target for therapeutic intervention.

The ability to control the adverse effects of IL-1 is furthered by the methods of treatment of this invention and the use of compounds thereby which inhibit IL-1 in mammals, specifically humans, who are in need of such use. There remains a need for compounds which are useful in treating IL-1 mediated disease states which are exacerbated or caused by the production of Interleukin -1 (IL-1).

SUMMARY OF THE INVENTION

This invention relates to a method of treating an IL-1 mediated disease state in a mammal in need thereof by administering to said mammal an effective amount of an inhibitor of mammalian mdr P-glycoprotein mediated IL-1 secretion, provided that the inhibitor is not a steroid or hormonal analog.

DETAILED DESCRIPTION OF THE INVENTION

There are presently known to be three members of the IL-1 family, IL-1α, IL-1β and IL-1ra. Il-1α and IL-1β are active moieties and are able to bind to receptors on target cells. In contrast IL-1ra does not have activity and yet has comparable affinity to the same receptors, indicating that it is a natural receptor antagonist to IL-1α and IL-1β.

The major source of IL-1 activity is the monocyte-macrophage cell, and the major form secreted is IL-1β. In monocytes, Il-1α and IL-1β are synthesized as 31 kDa precurser molecules which are subsequently cleaved to active, mature, carboxy terminal fragments upon release. Secretion of both precursors and mature forms has been observed, and yet precursor IL-1β is inactive, whereas precursor IL-1α is active.

There has been considerable controversy over the secretion pathway of IL-1. This is because both IL-1α and IL-1β lack the conventional hydrophobic peptide sequence at or near their amino terminus expected for translocation into the endoplasmic reticulum (ER) and secretion through the usual pathway. Studies have indicated that these proteins are made in the cytosol, and do not translocate into the ER. If a hydophobic signal sequence is attached to IL-1β and the

modified form expressed in monkey kidney cells (COS cells), it does go through the ER and gets secreted, and is in addition glycosylated. In contrast, natural IL-1β is not glycosylated. The query, therefore, is whether IL-1 is released from non-viable cells, ie., a non-active process, or whether the IL-1 is actively secreted.

An alternative pathway for secretion of IL-1 appears to have been looked at in bacteria and yeast, and which pathway does not require a hydrophobic signal sequence. The proteins secreted in yeast and bacteria have well defined extracellular activities, and range in size from the small yeast a-factor mating type peptide to the 53kDa bacterial hemolysin. A key feature of this pathway is the requirement for a membrane phosphoglycoprotein (P-glycoprotein) which is homologous in both yeast and bacteria. A major feature is a motif of six hydrophobic transmembrane domains followed by a cytosolic ATP binding region. In yeast this structure is duplicated suggesting that in the bacterial form it may function as a dimer. The bacteria and yeast proteins share the above noted structural features and possess amino acid similarity with other membrane proteins which transport amino acids, sugars and other small molecules.

The bacterial and yeast P-glycoproteins are somewhat similar to a series of mammalian P-glycoproteins originally identified for their ability to confer multidrug resistance when overexpressed in the host cell. Multidrug resistance is a clinically important obstruction to long term cancer treatment. Tumors treated with one form of chemotherapeutic agent often arise later in a resistant form. It has been observed that these tumor cells are not only resistant to the original drug, but also to other non-structurally related chemotherapeutic agents, thus restricting the available drugs to keep the disease in check. This resistance phenotype has been observed in vitro with cell lines, and has been shown to

be conferred by amplification of the multi-drug-resistance gene (hereinafter referred to as mdr) and overexpression of its P-glycoprotein product. Cloning has indicated that there are two highly related mdr genes in man, three in rodents, and at least one of the mdr1 genes has clearly been associated with multi drug resistance. P-glycoprotein seem to function by "pumping" the toxic drugs out of the cells, thus maintaining a lower concentration within the cells.

Due to its key role in drug resistance, there has been a tremendous effort to develop compounds which will inhibit the mdr "pump". One extensive review of Ford et al., Pharmacology of Drugs That Alter Multidrug Resistance in Cancer, Pharm. Rev. 42, pp 155-99 (1990) whose disclosure is incorporated by reference in its entirety herein. For greater detail concerning the specific biochemistry of P-glycoprotein mediated drug resistance see Endicott et al. Annu. Rev. Biochem. 58:137-71 (1989) whose disclosure is also incorporated by reference in its entirety herein. As a general statement, increased expression of the P-glyoprotein causes multidrug resistance. The basic question defining the specificity of the pump, how energy is transduced by the protein for active efflux is yet unanswered.

A number of structurally different compounds have been found to inhibit the pump. One of the earliest compounds identified was verapamil, a calcium channel antagonist. However, extensive structure activity work on this class of compounds has indicated that the ability to inhibit the mdr1 glycoprotein is not correlated with its ability to inhibit calcium channels. Generally the compounds which have been found to inhibit the mdr P-glycoprotein are

(i) calcium antagonists, such as but not limited to, verapamil, verapamil analogs, desmethoxyverapamil, dilitiazem, nifedipine, nifedipine analogs: tiapamil, DMDP, nicardapine, diludapine, nimodipine; dihyropyridine analogs,

caroverine, prenylamine, bepridil, AHC-52, Ro11-2933, perhexiline maleate, or SDB-ethylendiamine;

(ii) calmodulin antagonist, such as but not limited to, the phenothiazine derivatives: trifluoperazine, thioridazine, chlorpromazine, prochlorperazine, trifluropromazine, perphenazine, chlomiperamine, fluphenazine, flupenthixol, chlorprothiexene, clolpenthixol, W-12 or W-13.

(iii) noncytotoxic anthracyclines, Vinca alkaloids or Vinca alkaloid analog, such as but not limited to, ID-8279, Vindoline, daunorubicin, doxorubicin, cepharanthine, or aclacinomyin A;

(iv) cyclosporins, such as but not limited to, Cyclosporin A, Cyclosporin C, Cyclosporin G, Cyclosporin H, 11-Me-Ile (W8-032), O-Acetyl C_9, 11-Me-Leu, or 6-Me-Ala;

(v) hydrophobic cationic compounds which are amphipathic and lipophillic nature, contain a broad structural similarity that includes a heterocyclic ring nucleus seperated at a distance from a cationic, amino group; such as but not limited to dipyridamole, quinacrine, primaquine, quinidine, quinine, amiodarone, indole alkaloids - reserpine, rescinnamine, yohimibine, trimethoxybenzoyl-yohimibine; alkaloid derivatives of cepharanthine, lysosomotropic amines- chloroquine,propranol; erythromycin, cefoperazone, ceftriaxone, and triparanol,. .

Calmodulin antagonists as used herein are meant to include a group of anti-mdr agents which possess the ability to inhibit CaM-mediated processes, such as the Ca+2/CaM-dependent form of cyclic nucleotide phosphodiesterase. For greater detail, see Ford et al., Pharm. Reviews, Vol.42, No. 3 (1990) pages 155-199, specifically 168-173 inclusive, the entire contents of which are incorporated by reference herein. Preferred calmodiulin antagonists are the phenothiazine derivatives.

By the term "steroid or hormonal analogs" as used herein is meant agents which are generally referred to as a steroid, i.e. possesing a steriod ring nucleus and those hormonal analogs which posses antiestrogen activity, such as tamoxifen, toremifene structurally related triparanol analogs, which are referred to in Ford et al., Pharm. Reviews, Vol.42, No. 3 (1990) pages 155-199, specifically 168-173 inclusive,Ford et al., Pharm. Reviews, Vol.42, No. 3 (1990) pages 155-199, specifically p 173 inclusive.

The method of this invention may also include using an inhibitor of the P-glycoprotein IL-1 secretion as defined herein, co-administered with a steroid or hormonal analog.

IL-1 is a protein which is both produced and secreted. The present invention provides for a method of inhibiting the secretion of IL-1 by compounds which are inhibitors of the mammalian mdr P-glycoprotein "pump". As noted above interleukin - 1β is a protein which lacks weak hydrophobic sequence signals which are generally expected to be present to allow for translocation into the endoplasmic reticulum (ER) and thereby secretion by the usual pathway. As also noted above, previous work has indicated that these proteins are not translocated into the ER. The present invention demonstrates that the P-glycoprotein moiety is involved in active transport of IL-1 and inhibition of the mdr "pump" by an agent, would clearly provide a means for prophylactically treating, or ameliorating IL-1 mediated disease states, particularly those disease states which are strongly inflammatory oriented, such as arthritis.

In its simplest form, the present invention is a method for treating an inflammatory disease which is mediated by IL-1 , in a mammal, including humans, with any compound capable of inhibiting the mdr P-glycoprotein and is

administered in an amount sufficient to inhibit the P-glycoprotein.

The mammalian, and specifically human, mdr1 and mdr3 gene, its sequencing and their various P-glycoprotein compounds are well known to those skilled in the art. For additional information see Chen et al., Cell, 47, pp 381-389 (1986); see also McGrath et al., Nature, Vol. 340, pp 400-04 (1989) whose disclosures are herein incorporated by reference.

Interleukin-1 (IL-1) has been demonstrated to mediate a variety of biological activities thought to be important in immunoregulation and other physiological conditions such as inflammation [See, e.g., Dinarello et al., Rev. Infect. Disease, 6, 51 (1984)]. The myriad of known biological activities of IL-1 include the activation of T helper cells, induction of fever, stimulation of prostaglandin or collagenase production, neutrophil chemotaxis, induction of acute phase proteins and the suppression of plasma iron levels.

There are many disease states in which excessive or unregulated IL-1 production is implicated in exacerbating and/or causing the disease. These include rheumatoid arthritis, osteoarthritis, endotoxemia and/or toxic shock syndrome, other acute or chronic inflammatory disease states such as the inflammatory reaction induced by endotoxin or inflammatory bowel disease; tuberculosis, atherosclerosis, muscle degeneration, cachexia, psoriatic arthritis, Reiter's syndrome, rheumatoid arthritis, gout, traumatic arthritis, rubella arthritis, and acute synovitis. Recent evidence also links IL-1 activity to diabetes and pancreatic ß cells.

Dinarello, J. Clinical Immunology, 5 (5), 287-297 (1985), reviews the biological activities which have been

attributed to IL-1. It should be noted that some of these effects have been described by others as indirect effects of IL-1. See also Dinarello, Inflammation: Basic Principles and Clinical Coorelates, Ed. Galin et al., Raven Press, Ltd., New York, New York, pp 195-208, (1988) and references cited therein.

This invention further relates to the use of a compound as defined by this invention, or pharmaceutically acceptable salts thereof, in the manufacture of a medicament for the treatment of prophylactically or therapeutically, any disease state in an animal, including humans, which is exacerbated or caused by excessive or unregulated IL-1 production by inhibition of mammalian mdr P-glycoprotein IL-1 secretion.

By the term "mammalian mdr P-glycoprotein" as used herein is meant the naturally occuring P-glycoprotein moiety produced by any multi-drug-resistance genes, any variant or homolog thereof, as well as functional equivalents thereof which may or may not confer mdr to the host cells.

At present only two mdr genes are known, and are generally referred to as mdr1 and mdr3. Preferably the P-glycoprotein inhibited is the mdr1 expressed P-glycoprotein. One skilled in the art can readily determine whether or not a P-glycoprotein is functionally equivalent by recognized assays used to determine multi-drug resistance for instance the chemotherapeutic agents used for cancer chemotherapy.

These assays would include indicators such as decreased sensitivity to a particular drug by the cell in the presence of overexpressed mdr and/or an ability of the cell to increase the toxic levels of a drug by reducing the intracellular levels of that drug. Such assays can be found, for instance in, van Der Blick, et al., EMBO J. 6:3325-31

(1987) whose disclosure is incorporated by reference herein. See also Ford, et al., Pharmacology of drugs that alter multidrug resistance in cancer. Pharm. Rev. 42: 155-199 (1990), and Endicott et al., Annu Rev. Biochem., 58:137-71 (1989).

By the term "inhibiting the production of IL-1" is generally meant

a) a down regulation, by inhibition of the direct synthesis of IL-1 as a postranslational event; or

b) a decrease of excessive in vivo IL-1 levels in a human to normal levels or below normal levels by inhibition of the in vivo release of IL-1 by all cells, including but not limited to, monocytes or macrophages; or but least preferred is

c) a down regulation, at the transcription or translational level, of excessive in vivo IL-1 levels in a human to normal levels or below normal levels.

By the term "IL-1 mediated disease or disease state" is meant any and all disease states in which IL-1 plays a role, either by production of IL-1 itself, or by IL-1 causing another monokine in the cytokine cascade to be released, such as but not limited to TNF, or IL-6. A disease state in which TNF, for instance is a major component, and whose production or action, is exacerbated or secreted in response to IL-1, would therefore be considered a disease mediated by IL-1.

The term "monokine" is generally referred to as being a polypeptide produced and secreted by a mononuclear cell, such as a macrophage and/or monocyte but many other cells produce monokines, such as natural killer cells, fibroblasts, basophils, neutraphils, endothelial cells, brain astrocytes, bone marrow stromal cells, epideral

keratinocytes, and ß- lymphocytes. Lymphokines are generally referred to as being produced by lymphoctye cells.

By the term "IL-1 interfering or suppresive amount" is meant an effective amount of a compound of this invention which will, when given for the treatment, prophylactically or therapeutically, of any disease state which is exacerbated or caused by excessive or unregulated cytokine production, cause a decrease the in vivo levels of IL-1 or other cytokines/monokines, to normal or below normal levels.

METHODS OF USE

This invention also relates to the use of a pharmaceutical composition for use in treating an IL-1 mediated disease, comprising an effective amount of an inhibitor of P-glycoprotein , a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier or diluent. The compounds used in the process of this invention can be administered in conventional dosage forms prepared by combining an inhibitor as defined herein, with standard pharmaceutical carriers according to conventional procedures.

A wide variety of pharmaceutical dosage forms can be employed. The route of administration will depend upon the particular inhibitor chosen. It will be recognized by one of skill in the art that the form and character of the pharmaceutically acceptable carrier or diluent is dictated by the amount of active ingredient with which it is to be combined, the route of administration and other well-known variables.

The methods of the subject invention may be carried out by delivering the monokine activity interfering agent by various routes of administration , and includes such routes as oral, pulmonary, parenteral, buccal, intra-articular, nasal or topical. The term 'parenteral' as used herein includes intravenous, intramuscular, subcutaneous intranasal,

intrarectal, intravaginal or intraperitoneal administration. Subcutaneous and intramuscular forms of parenteral administration are generally preferred. Appropriate dosage forms for such administration may be prepared by conventional techniques.

The amount of an inhibitor as disclosed herein, required for therapeutic effect upon administration will, of course, vary with the compound chosen, the nature and severity of the inflammatory condition, the mammal undergoing treatment, and is ultimately at the discretion of the physician. It will also be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of an inhibitor of the present invention , or a pharmaceutically acceptable salt thereof will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the particular patient being treated, and that such optimums can be determined by conventional techniques.

It will also be appreciated by one of skill in the art that the optimal course of treatment, i.e., the number of doses of an inhibitor of the present invention or a pharmaceutically acceptable salts thereof given per day for a defined number of days, can be ascertained by those skilled in the art using conventional course of treatment determination tests.

EXAMPLES

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following Examples are, therefore, to be construed as merely illustrative and not a limitation of the scope of the present invention in any way.

UTILITY EXAMPLES

The present invention demonstrates that human mdr1 and mdr3 are involved in IL-1 secretion. The following assay, described below, uses COS (monkey kidney) cells co-transfected with DNA vectors encoding various combinations of precursor or mature IL-1β and human mdr1 or mdr3. In the absence of mdr, precursor and mature IL-1β are not secreted. In the presence of mdr1, mature IL-1β is secreted and precursor IL-1β is not. Human mdr3 is shows a small amount of secretion relative to the control and to mdr1.

These findings demonstrate that human mdr1 functions in IL-1 secretion. In addition to this showing, human mdr1 mRNA is also expressed in activated monocytes. The fact that precursor IL-1β is not secreted by mdr1 indicates a preference for the processing upon secretion, which reflects the biosynthesis data in activated monocytes.

This data clearly supports the use of "pump" inhibitors for treating an IL-1 mediated disease state.

Materials and Methods

Human monocytes

Peripheral blood monocytic cells were obtained after isolation from Red Cross buffy coats. The isolation procedure consisted of sequential Lymphoprep (Nyegaard, Oslo, Norway) and Percoll (Pharmacia) gradient centrifugation as described in Colatta et al., 1985. Purified monocytes were plated onto 100 cm^2 tissue culture dishes and allowed to adhere in the presence of RPMI (GIBCO) + 1% human AB serum (MA Bioproducts, Walkersville, MD) + 100 ng/ml bacterial lipopolysaccharide (*E. coli* 055:B5, Difco, Detroit, MI). One hour after plating, LPS-stimulated monocytic cells were metabolically labeled according to the protocol described below.

COS cells and transient transfection.

COS cells were maintained at 37 °C / 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 10 u/ml penicillin, 10 mg/ml streptomycin and 25 ng/ml amphotericin B. All tissue culture media and supplements, except where indicated, were supplied by GIBCO.

Transfection for transient expression in COS cells was carried out by a DEAE-dextran/chloroquine protocol. Cells were seeded on 100 cm^2 culture plates at 1.5 X 10^6 cells per plate 16 - 24 h before transfection. Just prior to transfection, cells were washed with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.3 mM KH_2PO_4, 9.5 MM Na_2HPO_4) and incubated for 1 h in 2.5 ml of DMEM containing 10% NuSerum (Collaborative Research, Lexington, MA). Cesium chloride gradient-purified plasmid DNA (20 mg per plasmid per transfection) was resuspended in 0.5 ml of Tris buffered saline (TBS; 137 mM NaCl, 5 mM KCl, 1.5 mM Na_2HPO_4, 250 mM Tris-HCl, pH 7.5, 1.4 mM $CaCl_2$, 1.1 mM $MgCl_2$) before it was added to 2 ml of DMEM containing 1 mg/ml DEAE-dextran (Pharmacia) and 1 mg/ml chloroquine (Sigma). The 2.5 ml of transfection mixture was then added to the 2.5 ml of DMEM/NuSerum on the cells. The cells were incubated 2.5 h in the presence of the transfection mixture before being washed with PBS, shocked with 10% DMSO in PBS for 3 min, washed again with PBS and incubated 42 - 48 h in DMEM/10% FBS/antibiotics/antimycotic as described above. Mock transfectants were generated as above, but in the absence of exogenous DNA.

Metabolic labeling, verapamil treatment, and immunoprecipitation

Transfectants 42 - 48 h post-DMSO shock were washed with PBS before depleting them of internal cysteine and methionine pools by incubating them for 1 h in cysteine- and methionine-deficient DMEM containing 1% dialyzed FBS (dFBS). Cells were

then radiolabeled by incubating them with fresh cysteine- and methionine-deficient DMEM/1% dFBS (dialyzed FBS) containing 150 mCi/ml (with respect to methionine) Tran^{35}S-label (ICN) for 1 h. Secreted products were allowed to accumulate in chase medium (DMEM/1% FBS) for various times following the labeling period. Cells treated with verapamil (verapamil hydrochloride, Sigma V-4629) were incubated with the drug during both the labeling and chase periods. LPS-stimulated monocytic cells (1 h post-plating) were labeled and treated similarly, except that 100 ng/ml LPS was present at all times.

Labeled cells were harvested by removing the media, washing with PBS and lysing on the plate with 1 ml of RIPA buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM EDTA, 2 mM PMSF). Sample aliquots were cleared prior to immunoprecipitation by making them 1X RIPA/2% Carnation milk (lysate samples) or 0.5X RIPA/0.5% milk (media samples) and rotating them for 0.5 h at 4 °C after adding 15 µl of half-packed Protein A - Sepharose 4B beads (Pharmacia). Beads were pelleted by centrifugation at 2000 X g. Cleared samples were transfered to fresh tubes and incubated for 16 h at 4 °C with a rabbit anti-human IL-1bβ antiserum S32 (Hazuda et al., Biol. Chem., 263:8473-8479 (1988)) or a rabbit anti-rat ATP citrate lyase antiserum. Immune complexes were recovered by adding 50 ml of half-packed Protein A - Sepharose 4B beads to the samples, rotating the samples for 1 h at 4 °C and pelleting the beads as described above. The beads were washed three times with 1X RIPA buffer before resuspending them in either 50 ml of SDS-PAGE gel loading buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 8% glycerol, 1.2 M b-mercaptoethanol, 0.1% bromphenol blue). Prior to size-fractionation of immunopreciptated products on SDS-PAGE gels (Laemmli, Nature, 227:680-685 1970), the immune complexes were separated from the beads by boiling the samples for 5 min and pelleting the beads as described above. Gels were fixed by soaking them in two

washes (15 min each) of 10% acetic acid/30% methanol. Prior to drying, gels were treated with a fluor (Amplify, Amersham) for 15 min. Autoradiography was carried out at -70 °C using Kodak XAR film and a DuPont Cronex Lightning Plus intensifying screen.

Plasmids

The plasmids directing the expression of human IL-1β, RSVIL1b Precursor and RSVIL1b Mature, were constructed from RSVCATBGH by deleting the 726 bp Hind III/Xba I fragment containing the bacterial chloramphenicol acetyl-transferase gene and replacing it with either the 950 bp Hind III-partial/Xba I fragment of DSPIL-1β precursor (Young et al., J. Cell Biol., 107:447-456, 1988) or the 1.1 kb Hind III-partial/Nhe I fragment of DSPIL-1β mature. [DSPIL-1β mature was generated by ligating the 1.1 kb Nco I/Xba I fragment of pMGNcoIL-1β into DSP1 (See Pfarr et al., DNA 4:461-467 1985; Lillquist et al., 1988, J. Immunol. 141:1975-1981; Meyers et al., 1987, J. Biol. Chem. 262:11176-81). The plasmids directing the expression of human multiple drug resistance genes mdr1 and mdr3, pJ3Wmdr1.1 and pJ3Wmdr3, See van Der Blick et al., EMBO J., 6:3325-31 (1987). The plasmid directing the expression of human ATP citrate lyase, HCL-RJB2, can be readily constructed by one skilled in the art using the literature references of Elshourbagy et al., 1990, J.Biol Chem., 265:1430-35, an article disclosing rat ATP citrate lysase cDNA sequence and for the vector RJB2 see Caltiabiano et al., GENE, 85:479 (1989).

Standard recombinant DNA manipulations were carried out as described in Sambrook et al. Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1989). Insert/vector junction regions were confirmed by dideoxy DNA sequencing with Sequenase (United States Biochemical Corporation).

Drug efflux assay

COS cells transfected with RSVIL1b Mature or co-transfected with RSVIL1b Mature and either pJ3Wmdr1.1 or pJ3Wmdr3 were split 1:1 onto fresh 100 cm^2 tissue culture plates immediately following trypsinization one day after transfection. One day following the split, one plate of each sample (approximately $1x10^6$ cells) was metabolically labeled and immunoprecipitated with antiserum S32 to assess mature IL-1β production and secretion as described above. The parallel samples, representing the remaining half of the cells replated the previous day, were washed once with PBS before doping for 20 min with 5mCi of ^3H-vinblastine sulphate (Amersham) in 4 ml of DMEM/1% FBS. Cells were then washed twice in PBS before incubating for 20 min in 4 ml of DMEM/1% FBS to allow released vinblastine to accumulate in the medium. Media was collected and the cells lysed as described above. The entire volume of media and cell lysates were analyzed by scintillation analysis in a Beckman LS 7800 scintillation counter using Beckman Ready Safe scintillation fluid as a fluor.

RNA preparation and Northern analysis

COS transfectants used for the preparation of RNA were split 1:1 onto fresh 100 cm^2 tissue culture plates immediately following trypsinization one day after transfection. One day following the split, one plate of each sample (approximately $1x10^6$ cells) was metabolically labeled and immunoprecipitated with antiserum S32 to assess mature IL-1β production and secretion as described above. The parallel samples, representing the remaining half of the cells replated the previous day, were washed once with PBS before being incubated for 30 min in cysteine- and methionine-deficient DMEM/1% dFBS. Cells were then incubated for 4 h in DMEM/1% FBS before preparing total RNA by a guanidinium lysis/cesium chloride gradient purification protocol, See Sambrook et al, supra. Twenty to 30 mg of each

sample was run on a 1.0% agarose/2.2 M formaldehyde gel as described in Sambrook et al., supra (1989). RNA was transferred to a Hybond-N nylon membrane (Amersham) and UV irradiated for 5 min with an Ultraviolet Products UV transilluminator. Membranes were then pre-hybridized for 2 h and hybridized for 16 h in a 50% formamide solution (See Elshourbagy et al., 1990, J.Biol Chem., 265:1430-35.) at 57 ^{0}C. Membranes were washed for approximately 1 h in 0.2X SSC/0.2% SDS at 65 ^{0}C (0.2X SSC = 30 mM sodium chloride, 3 mM sodium citrate). The probe used was 0.5 mg of the 4.0 kb Xba I insert from pJ3Wmdr1.1 nick-translated with 100 mCi of ^{32}P-dCTP using an Amersham N.5000 nick-translation kit. The probe was separated from unincorporated deoxynucleotides by filtration over a G-50 column. Prior to hybridization, the probe was boiled for 10 min and iced for 5 min.

Human peripheral blood monocytic cells for RNA preparation were treated immediately after isolation by plating onto T175 tissue culture flasks with 10 ml of RPMI/1% human AB serum. Immediately after plating, LPS-stimulated samples were treated with 100 ng/ml of LPS for 3 h. RNA was prepared 3 h after plating and analyzed as described above.

Isolation of mdr-like sequences by polymerase chain reaction from an LPS-stimulated human monocyte cDNA library

Oligonucleotide primers for PCR amplification were made to the well-conserved 3' (C-terminal) nucleotide binding site. The 5' primer (where I=inosine) is GGGCCGAATTCGCI (T/C)TIGT(G/C)GGI(T/A)(G/C)(C/T)(T/A) (G/C)(C/T)GGITG(C/T)GGIAA. The 3' primer is GGGCCGTCGAC(G/A)TCIA(G/A)IGCIGAIGTIGC(C/T)TC (G/A)TC. In addition to the terminal SmaI half-sites, 5' EcoRI site and 3' SalI for cloning, the mdr sequences represented in the primers correspond to 5' (N-terminal) ALVGSSGSGK and 3' (C-terminal) DEATSALD. The amplification reaction was carried out in a Perkin Elmer Cetus Thermal Cycler under the following conditions: five cycles of 94 ^{0}C for 30 sec, 45 ^{0}C

for 60 sec with a 10 sec extension per cycle, 72 ^{0}C for 60 sec; then, 35 cycles of 94 ^{0}C for 30 sec, 48 ^{0}C for 30 sec, 72 ^{0}C for 60 sec. The incubations were terminated after a 7 min incubation at 72 ^{0}C. The reaction contained 1 µg of each primer and 1 ng of DNA from an LPS-stimulated human monocyte library in a PBR322 background, See Meyers et al., J. Biol. Chem. (1987) 262:1176-1181; for a 1ng of DNA from a GM-CSF stimulated monocyte library in a lamdagt11 background, See Balcerak et al., 1988, J. Biol. Chem, 263:13937-13941. Only a single band, of approximately 440 bp, was visualized on an agarose gel. The DNA from this band was isolated, digested with EcoRI and SalI, and cloned into the EcoRI and SalI sites of the Bluescript KS+ vector. The resulting clones were sequenced as described above.

RESULTS

To address the non-endoplasmic reticulum-directed pathway of IL-1 secretion, various possible mechanisms of IL-1 release from both monocytic cells (the primary producers of secreted IL-1) and non-monocytic cells have been examined.

Test data demonstrates that human mature IL-1β (the β form of IL-1 being the predominant IL-1 species produced by human monocytic cells) is released from COS (non-monocytic) kidney cells transfected with RSVIL1β Mature by nonspecific leakage of cytosolic contents and not by a specific secretory mechanism. This release occurs only after long-term (22 h) incubation of the cells following metabolic labeling. Furthermore, human IL-1β precursor fails to be released into the media even after long-term incubation, apparently due to its short intracellular half-life (3 h).

The role of mdr-encoded proteins in IL-1 secretion was examined by the ability of two human mdr-encoded P-glycoproteins which permitted COS cells to secrete IL-1. COS cells were co-transfected with all four combinations of

plasmids expressing precursor or mature IL-1β, and mdr1 or mdr3. These transfectants were then subjected to pulse-chase analysis. This analysis showed that the IL-1β precursor failed to be efficiently released from cells co-transfected with either of the two mdr-encoding plasmids. Likewise, mature IL-1β failed to be efficiently released from COS cells co-transfected with pJ3Ωmdr3. However, mature IL-1β was efficiently released from cells expressing both mature IL-1β and mdr1.

As has been observed in LPS-stimulated monocytic cells almost the entire amount of mature IL-1β which is secreted from these co-transfected COS cells is released during the first four hours of the chase period. The release of mature IL-1β is apparently not due to nonspecific leakage of cytosolic proteins since i) ATP citrate lyase, a cytosolic protein, is not leaked into the media from COS cells expressing human ATP citrate lyase, mature IL-1β and mdr1 during the period of mature IL-1β secretion, and ii) COS cells expressing both mature IL-1β and mdr3 or mature IL-1β alone do not secrete mature IL-1β.

In inhibiting P-glycoprotein-mediated secretion of IL-1, both LPS-stimulated human monocytes and COS co-transfectants expressing both mature IL-1β and mdr1 were treated with Verapamil. Verapamil is a calcium channel antagonist which also inhibits the transport of various lipophilic, cationic chemotherapeutic drugs from several cell types which exhibit the multiple drug resistance phenotype (Ford et al, Pharm. Reviews, Vol.42, No. 3 (1990)). Results indicate inhibition of IL-1β secretion by verapamil, although some variability in dose response was observed, thereby giving positive affirmation that the "pump" mechanisms are inhibited and involved in IL-1 secretion. Additional examples clearly indicate, as noted above, that in the absence of mdr, precursor and mature IL-1β are not secreted. In the presence

of mdr1, mature IL-1β is secreted and pre-cursor IL-1β is not.

Although verapamil and other calcium channel antagonists bind P-glycoprotein transporters and interfere with their ability to export chemotherapeutic drugs from certain cells, it is clear that these agents do not inhibit drug export in all cases (Ford et al., Pharm. Reviews, Vol.42, No. 3 (1990).

The present results suggest that that inhibiting the IL-1β and/or general transport activities of the human mdr1-encoded P-glycoprotein with known or novel agents is beneficial for indications in which excessive extracellular IL-1 activity is implicated by providing a novel mechanism through which deleterious extracellular IL-1 activity can be abrogated.

The above description fully discloses the invention including preferred embodiments thereof. Modifications and improvements of the embodiments specifically disclosed herein are within the scope of the following claims. Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. Therefore the Examples herein are to be construed as merely illustrative and not a limitation of the scope of the present invention in any way. The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows.

CLAIMS

What is claimed is:

1. A method of treating an IL-1 mediated disease state in a mammal in need thereof by administering to said mammal an effective amount of an inhibitor of mammalian mdr P-glycoprotein mediated IL-1 secretion, provided that the inhibitor is not a steroid or hormonal analog.

2. The method of Claim 1 wherein the inhibitor is a calcium channel blockers.

3. The method of Claim 1 wherein the inhibitor is a calmodium antagonist.

4. The method of Claim 1 wherein the inhibitor is a noncytotoxic anthracyclines, a Vinca alkaloid or a Vinca alkaloid analog.

5. The method of Claim 1 wherein the inhibitor is a cyclosporin.

6. The method of Claim 1 wherein the inhibitor is a hydrophobic cationic compounds.

7. The method of Claim 2 wherein the inhibitor is administered with a steroid or hormonal analog.

8. The method of Claim 2 wherein the calcium channel blocker is selected from verapamil, tiapamil, dilitiazem, nifedipine, diludapine, nimodipine, dicardapine, dihyropyradine analogs, caroverine, prenylamine, bepridil, or SDB-ethylendiamine.

9. The method of Claim 3 wherein the calmodulin antagonist is selected from trifluoperazine, thioridazine,

chlorpromazine, prochlorperazine, trifluro-promazine, perphenazine, chlomiperamine, fluphenazine, flupenthixol, chlorprothiexene, or clolpenthixol.

10. The method of Claim 5 wherein the cyclosporin is selected from Cyclosporin A, Cyclosporin C, Cyclosporin G, Cyclosporin H, 11-Me-Ile, O-Acetyl C_9, 11-Me-Leu, or 6-Me-Ala.

11. The method of Claim 6 wherein the hydrophobic cationic compound is selected from dipyridamole, quinacrine, primaquine, quinidine, quinine, amiodarone, cepharanthine, chloroquine,propranol; erythromycin, cefoperazone, ceftriaxone, or triparanol.

12. The method according to Claim 1 wherein the IL-1 mediated disease is selected from athritis, rheumatoid arthritis, osteoarthritis,traumatic arthritis, rubella arthritis, acute synovitis, endotoxemia, toxic shock syndrome, Crohn's disease, ulcerative colitis, inflammatory bowel disease, tuberculosis, atherosclerosis, muscle degeneration, cachexia, psoriatic arthritis, Reiter's syndrome, gout, asthma, chronic pulmonary inflammatory diseases,.adult respiratory distress syndrome, or diabetes.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02214

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61 K 37/02
US CL : 514/9, 249, 290, 2

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	514/9, 249, 290, 2

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
A	US, A, 4,975,467 (Ku et al) 04 December 1990, see the entire document	1-12
A	US, A, 4,861,794 (Otterness) 29 August 1989, see the entire document.	1-12
A,P	US, A, 5,039,695 (Parker et al) 13 August 1991, see the entire document.	1-12
Y	US, A, 4,902,685 (Skotnicki) 20 February 1990, see col. 1, lines 56-68 and col. 2, lines 38-46.	1,12
Y	US, A, 4,208,414 (Schinitsky) 17 June 1980, see col. 2, lines 16-22.	1,4,12
Y,E	US, A, 5,114,951 (King) 19 May 1992, see col. 2, lines 26-35, col. 3, lines 56-68 and col. 4, lines 1-2 and lines 7-10.	1-4,8
A	US, A, 4,778,806 (Bender et al) 18 October 1988, see the entire document.	1-12

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
02 JUNE 1992	29 JUN 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	T. D. WESSENDORF LG

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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Agents and Actions, Vol. 21, issue nos. 3/4, issued 1987, DiMartino et al, "Effect of Anti arthritic drugs on the enhanced interleukin-1 (IL-1) production by macrophages from adjuvant-induced arthritic (AA) rats", pp. 348-350, see page 348, col. 1 and page 349, Table 2.	1,6,11,12
X	Agents and Actions, Vol. 21, issue nos, 3/4, issued 1987, Arner et al, "Effect of anti inflammatory drugs on human interleukin-1- induced cartilage degradation, pp. 334-336, see page 334, the Abstract and page 336, col. 2.	1,6,11,12

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
X	Agents and Actions, Vol. 25, issue nos. 1/2, issued 1988, Connolly et al, "Alteration of interleukin-1 activity and the acute phase response in adjuvant arthritic rats treated with disease modifying antirheumatic drugs", pp. 94-105, see page 97, col. 2.	1,5,6 and 10-12
X	Agents and Actions, Vol. 33, issue nos. 3/4, issued 1991, Hom et al, "Interleukin 1 mediated acceleration of type II collagen-induced arthritis: Effects of anti-inflammatory or anti-arthritic drugs", pp. 300-309, see page 304, Table 2.	1,5,12
A	Nature, vol. 340, issued 03 August 1989 McGrath et al, "The Yeast STE6 gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein", pp. 400-404, see the entire document.	1-12